Biochemical and Biophysical Research Communications **257**, 561–566 (1999)
Article ID bbrc.1999.0505, available online at http://www.idealibrary.com on IDEAL®

Exhibit 6

The Yeast Npi1/Rsp5 Ubiquitin Ligase Lacking Its N-Terminal C_2 Domain Is Competent for Ubiquitination but Not for Subsequent Endocytosis of the Gap1 Permease

Jean-Yves Springael, Johan-Owen De Craene, and Bruno André[1]
Laboratoire de Physiologie Cellulaire et de Génétique des Levures, Université Libre de Bruxelles-Campus Plaine CP244, Bd du triomphe, B-1050 Bruxelles, Belgium

Received March 1, 1999

The yeast ubiquitin ligase Npi1/Rsp5 and its mammalian homologue Nedd4 are involved in ubiquitination of various cell surface proteins, these being subsequently internalized by endocytosis and degraded in the vacuole/lysosome. Both enzymes consist of an N-terminal C_2 domain, three to four successive WW(P) domains, and a C-terminal catalytic domain (HECT) containing a highly conserved cysteine residue involved in ubiquitin thioester formation. In this study, we show that the conserved cysteine of the HECT domain is required for yeast cell viability and for ubiquitination and subsequent endocytosis of the Gap1 permease. In contrast, the C_2 domain of Npi1/Rsp5 is not essential to cell viability. Its deletion impairs internalization of Gap1, without detectably affecting ubiquitination of the permease. This suggests that Npi1/Rsp5 participates, via its C2 domain, in endocytosis of ubiquitinated permeases. © 1999 Academic Press

Internalization of plasma membrane proteins by endocytosis, followed by degradation in the lysosome, is a critical pathway for adaptation of eukaryotic cells to environmental changes. In *Saccharomyces cerevisiae*, for instance, endocytosis of the receptor of mating pheromone α (Ste2p) is constitutive, and enhanced several-fold upon binding of the receptor's ligand [1]. In yeast, accelerated internalization followed by vacuolar degradation is also a major mechanism for controlling the activity of several plasma membrane transporters in response to stress or nutritional changes [2; 3; 4; 5]. A critical step in endocytosis of many of these plasma membrane proteins is their prior conjugation to ubiquitin (reviewed in [6]). Studies on a truncated form of Ste2p have shown that binding of a single ubiquitin molecule promotes efficient receptor internalization [7]. In contrast, formation of lysine-63-linked polyubiquitin chains is required for maximal-rate internalization of both the uracil permease (Fur4) [8] and the general amino-acid permease (Gap1) [26]. The ubiquitination of Fur4 [9], Gap1 [3], the maltose permease (Mal61) [10] and probably many other plasma membrane proteins is defective in *npi1* mutant cells. This mutant displays severely reduced synthesis of Npi1/Rsp5 [11; 3], a ubiquitin-protein ligase [12] essential to cell viability [11]. The *S. cerevisiae* ubiquitin ligase Npi1/Rsp5 is homologous to Nedd4 of mammalian cells. Rat Nedd4 interacts with the epithelial Na^+ channel (ENaC), a multisubunit complex. The channel is ubiquitinated *in vivo*, this causing the number of channels in the plasma membrane to decrease [13; 14]. The Npi1 and Nedd4 ubiquitin ligases consist of an N-terminal C_2 domain (a widespread protein module interacting with diverse molecules including Ca^{++}, phospholipids, proteins, and inositol polyphosphates [15]), followed by three to four WW(P) domains (a protein motif binding to proline-rich sequences [16]), then a C-terminal catalytic domain (HECT) shared by many other ubiquitin ligases lacking the C_2 and WW(P) motifs (Fig. 1) [11; 12].

The central role of ubiquitin in down-regulation of many cell surface proteins has led to the general view that ubiquitin serves as a signal for endocytosis [6]. It might, for instance, be recognized by components of the endocytosis machinery, as yet unidentified. Recent data suggest that some proteins of the ubiquitin and endocytosis pathways may be associated in a complex genetic and two-hybrid interactions have been reported between the Npi1/Rsp5 ubiquitin ligase and Pan1, the yeast homologue of mammalian Eps15 [17]. The Pan1 protein is required for endocytosis [18] and interacts through distinct protein-binding domains with several proteins including End3p, an actin-regulating protein also required for endocytosis [19]; yAP180A, a yeast homologue of a class of clathrin

[1] To whom correspondence should be addressed. Fax: 32-2-650.54.21. E-mail: bran@ulb.ac.be.

0006-291X/99 $30.00

assembly proteins (AP180) [18], Sjl1p, an inositol polyphosphate 5-phosphatase related to synaptojanin [27]. It has been proposed that Pan1, through its interaction with these multiple factors, coordinates several activities, including ubiquitination, to ensure orderly and efficient endocytosis [18].

In this study we have investigated the role of the different domains of Npi1 in NH_4^+-triggered down-regulation of the Gap1 permease. We show that a truncated Npi1 protein lacking the C_2 domain can still promote ubiquitination but not subsequent endocytosis of Gap1. This is consistent with direct participation of Npi1, via the C_2 domain, in endocytosis of ubiquitinated permease.

MATERIALS AND METHODS

The *S. cerevisiae* strains used in this study, all isogenic with Σ1278b [25] except for the mutations mentioned, are 23346c (*MATa, ura3*); 27038a (*MATa, ura3, npi1*); 27039 (*MATa/MATa, ura3/ura3, leu2/leu2, NPI1/npi1Δ::LEU2*). Media, cell cultures, and [^{14}C]citrulline uptake assays are described in [3]. Crude and membrane-enriched yeast cell extracts were prepared and Western immunoblotting was performed as described in [3]. All *npi1* mutations were introduced into *NPI1* by site-directed mutagenesis by means of the Altered Site *in vitro* Mutagenesis System (Promega). To this end, a 6.5-kb *Eco*RI-*Sac*I fragment containing the *NPI1* gene was isolated from plasmid YCpJYS-1 [11] and inserted into the pALTER phagemid. Subsequent steps were performed as recommended by the manufacturer, using the following 5′ phosphorylated oligonucleotides: Npi1ΔC2, 5′-AAAATGCCTTCATCCATATCCGTCAAGTTAACTGCTACATCGAGTGGTAGACCTCGCGAA-3′; Npi1ΔWWP, 5′-AGACAATACTCTTCGTTTGAAGACCAGTATCCATCATCGCTAGACCAAAATGTTCCACAA-3′; Npi1ΔCys, 5′-TCTCACACATCTTTTAACAGA-3′. The mutagenized fragments were completely sequenced and the *Eco*RI-*Sac*I fragments bearing the correct mutations were introduced into the centromere-based plasmid pFL38 to yield plasmids YCp*npi1ΔC2*, YCp*npi1ΔWWP*, and YCp*npi1ΔCys*, or into the 2 μm-based plasmid to yield plasmids YEp*npi1ΔC2*, YEp*npi1ΔWWP*, and YEp*npi1ΔCys*.

RESULTS AND DISCUSSION

The Npi1/Rsp5 ubiquitin ligase is essential to cell viability, although why *npi1Δ* mutants are not viable remains unknown [11]. To investigate the role of the various domains of the protein in its essential *in vivo* function and in NH_4^+-induced down-regulation of the Gap1 permease, we constructed by site-directed mutagenesis three mutant proteins, represented schematically in Fig. 1A: Npi1ΔC_2 lacks the N-terminal C_2 domain, Npi1ΔWWP lacks a region spanning the three WW(P) domains, and in Npi1ΔCys the invariant cysteine of the HECT domain has been replaced with a serine. We tested the stability of these mutant proteins by Western blot immunodetection, using antibodies against mNedd4 [20], the mouse homologue of Npi1 (Fig. 1B). In wild-type cells these antibodies recognized two polypeptides, one at ~80 kDa and one at ~90 kDa (lane 1). We have previously shown that the 90-kDa

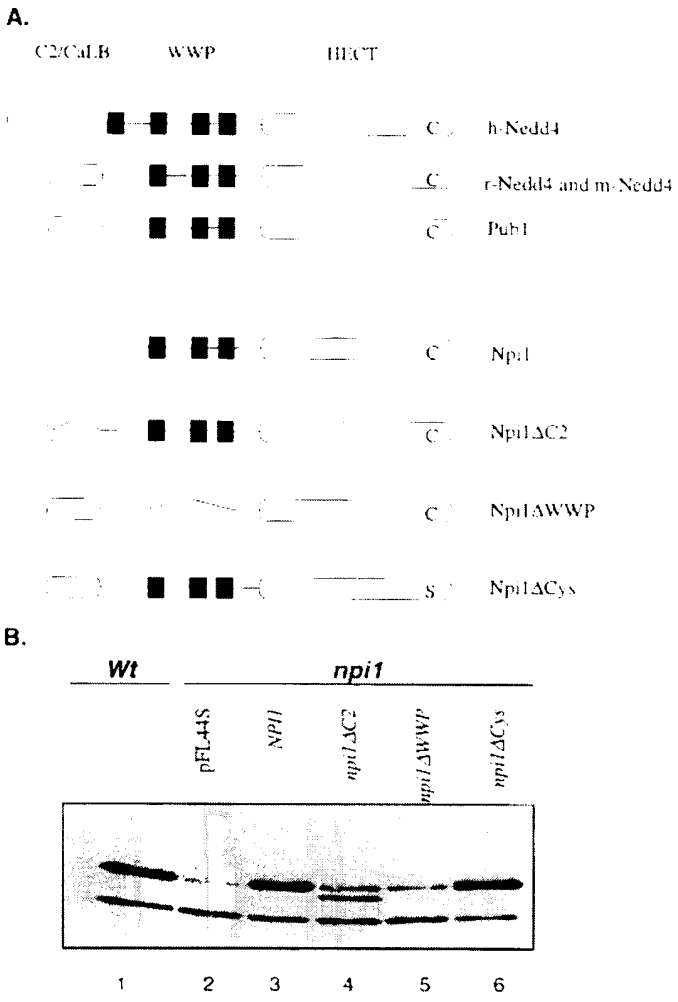

FIG. 1. (A) Schematic representation of the *S. cerevisiae* Npi1/Rsp5 ubiquitin ligase and its homologues in man (h-Nedd4), rat (r-Nedd4), mouse (m-Nedd4), and the yeast *S. Pombe* (Pub1). The mutant Npi1 proteins generated by site directed mutagenesis are also shown: Npi1ΔC_2 (deletion of amino acids 11-101), Npi1ΔWWP (deletion of amino acids 229-420), and Npi1ΔCys (replacement of cysteine 777 with a serine). Deleted regions are indicated by joined oblique lines. (B) Immunodection of wild-type and mutant Npi1 proteins in crude yeast-cell extracts. Crude extracts were prepared from proline-grown cells, resolved by SDS-Page, blotted onto a nitrocellulose membrane, and probed with polyclonal antibodies against mNedd4. The strains used were 23346c (wild type) (lane 1) and 27038a (*npi1*) transformed with plasmid pFL44S without insert (lane 2) or bearing the *NPI1* (lane 3), *npi1ΔC2* (lane 4), *npi1ΔWWP* (lane 5), or *npi1ΔCys* (lane 6) gene.

polypeptide is the ubiquitin ligase Npi1 since the intensity of the corresponding band decreased at least 10-fold in viable *npi1* cells (displaying severely reduced expression of the *NPI1* gene due to upstream insertion of a Ty element) (lane 2) and is restored to normal upon transformation of *npi1* cells with a plasmid bearing the wild-type *NPI1* gene (lane 3) [3; 11]. Cells of the *npi1* mutant were also transformed with plasmids bearing

the *npi1ΔCys*, *npi1ΔC*$_2$, and *npi1ΔWWP* genes. In *npi1ΔCys* transformants, the 90-kDa signal was normal, indicating that Npi1ΔCys is a stable protein (lane 6). The partially deleted Npi1ΔC$_2$ protein appeared as a third band of the size (84-kDa) expected for an Npi1 lacking its C$_2$ domain (lane 4). Finally, no additional band was detected in *npi1ΔWWP* transformants (lane 5), indicating that Npi1ΔWWP is either unstable or undetectable under our experimental conditions.

To assess the contribution of the C$_2$ domain and HECT-domain cysteine residue to the essential *in vivo* function of Npi1, a diploid strain with heterozygous deletion of *NPI1* gene was transformed with either the wild-type *NPI1* gene or the mutant genes *npi1ΔCys* and *npi1ΔC*$_2$. As expected, four viable spores were obtained after sporulation of diploid cells transformed with *NPI1*. Only two viable spores were obtained from tetrads derived from *npi1ΔCys*-transformed cells; four were obtained from tetrads derived from *npi1ΔC2*-transformed cells. These results indicate that the cysteine residue within the HECT domain, but not the N-terminal C$_2$ domain, is required for the essential *in vivo* function of Npi1. During the preparation of this manuscript, Wang *et al.* [28] reported similar results. For an unknown reason, the viable *npi1* mutant grows very slowly on minimal medium containing L-serine as the sole nitrogen source. The mutant *npi1ΔC*$_2$ gene could restore normal growth on this medium, while the *npi1ΔCys* gene could not (data not shown). It is known that the invariant cysteine of the HECT domain binds ubiquitin, and that the resulting ubiquitin-thioester is an obligatory intermediate in Npi1/Rsp5-catalyzed ubiquitination [12]. Hence, both the non-viability of *npi1Δ* mutants and the slow growth on serine of viable *npi1* mutants are most likely due to loss of Npi1-associated ubiquitin ligase activity. In contrast, since the C$_2$ domain is required neither for cell viability nor for growth on serine, its function must be non-essential and either unrelated to ubiquitination or required for ubiquitination of only some Npi1 substrates.

We next compared the ability of Npi1, Npi1ΔCys, and Npi1ΔC$_2$ to mediate NH_4^+-induced ubiquitination and subsequent down-regulation of the Gap1 permease. In these experiments, *npi1* cells transformed with a low-copy-number plasmid bearing *NPI1*, *npi1ΔCys*, or *npi1ΔC*$_2$ were grown on minimal proline medium, then NH_4^+ was added to each culture (defining time zero). At intervals, Gap1 activity was measured (Fig. 2A) and the stability (Fig. 2B) and ubiquitination state (Fig. 2C) of pre-synthesized Gap1 were assessed by immunoblotting of crude and membrane-enriched cell extracts respectively, using polyclonal anti-Gap1 antibodies. To detect ubiquitinated Gap1 forms, it was necessary to overexpose immunoblots of membrane-enriched preparations [3]. The Gap1 signal detected in proline-grown *npi1* cells transformed with the *NPI1*-bearing plasmid consists of a doublet around 60 kDa and of minor bands of higher molecular weight (Fig. 2C). Previous work has shown that these minor upper bands correspond to ubiquitin conjugated forms of the permease [3]. After addition of NH_4^+, the amount of ubiquitin-conjugated Gap1 was found to increase (Fig. 2C), both Gap1 activity and the Gap1 immunodetection signal decreased rapidly (Fig. 2A, B), reflecting internalization and subsequent vacuolar degradation of the permease. In contrast, no minor upper bands appeared in the Gap1 signal detected in *npi1* cells (Fig. 2C), and after NH_4^+ addition, both Gap1 activity and the Gap1 signal remained stable (Fig. 2A, B). Transforming *npi1* cells with the *npi1ΔCys*-bearing plasmid did not alter this phenotype (Fig. 2), indicating that the invariant cysteine within the HECT domain is required for *in vivo* ubiquitination and subsequent down-regulation of the Gap1 permease. A different phenotype was observed with *npi1* cells transformed with the *npi1ΔC*$_2$-bearing plasmid: although Gap1 again remained active (i.e., located in the plasma membrane) and strongly protected against degradation after NH_4^+ addition (Fig. 2A, B), the minor bands indicative of Gap1 ubiquitination did clearly appear, in addition to the main Gap1 signal (Fig. 2C). The ubiquitinated forms were already visible in proline-grown cells, increasing in intensity upon addition of NH_4^+. The low level of full-length Npi1 enzyme present *npi1* cells (Fig. 1B) cannot explain the appearance of these bands, since expression of Npi1ΔC$_2$ in *npi1Δ* cells produced the same pattern of Gap1 ubiquitination (data not shown). Deletion of the Npi1 C$_2$ domain thus renders Gap1 resistant to NH_4^+-induced down-regulation, without preventing ubiquitination of the permease. We tested the possibilty that Npi1ΔC$_2$ enzyme might ubiquitinate Gap1 less effectively than Npi1, the difference being hardly detectable in immunoblot experiments though sufficient to protect Gap1 against NH_4^+-induced down-regulation (Fig. 2C). We transformed *npi1* cells with a multiple-copy-number plasmid bearing the *NPI1* or *npi1ΔC*$_2$ gene and compared Gap1 sensitivity to NH_4^+ regulation in these transformants (Fig. 3). Gap1 signals were quantitated on immunoblots after different exposures. Before and five minutes after NH_4^+ addition, the proportion of ubiquitinated Gap1 forms did not differ significantly between Npi1- and npi1ΔC$_2$-overexpressing cells (Fig. 3C). After NH_4^+ addition, Gap1 was clearly down-regulated in the former but remained active and protected against degradation in the latter (Fig. 3A, B). These results indicate that Npi1ΔC$_2$ is competent for ubiquitination but not for subsequent down-regulation of Gap1. Interestingly, addition of NH_4^+ to cells overexpressing Npi1ΔC$_2$ did not lead to complete conversion of Gap1 to ubiquitinated forms. Rather, the relative amount of these forms remained limited and constant until one hour after NH_4^+ addition (Fig. 3C). A similar behavior has been described for the actin mutant (*act1-1*) defective in endocytosis and for the *gap1*^{ts}

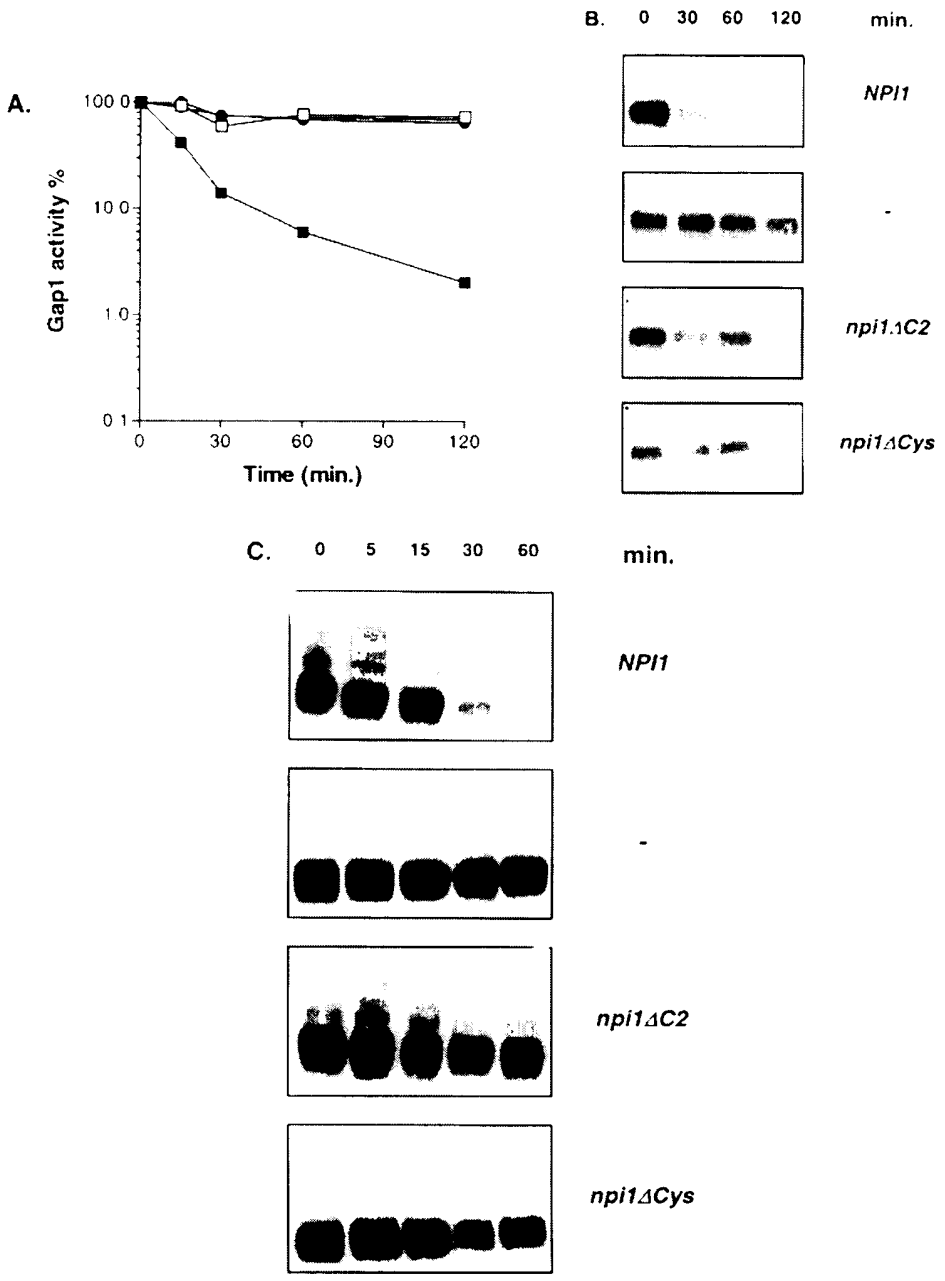

FIG. 2. Ubiquitination and down-regulation of Gap1 permease in cells expressing wild-type and mutant forms of the Npi1 ubiquitin ligase. (A) Cells were grown on minimal proline medium and Gap1 activity was measured by incorporation of [^{14}C]citrulline (0.02 mM) before addition of 10 mM $(NH_4)_2SO_4$ (t_0) and at intervals thereafter. Strains 27038a (*npi1*) transformed with the low-copy-number plasmid pFL38 without insert (□) or bearing the wild-type *NPI1* (■) or mutant *npi1*Δ*C2* (●) or *npi1*Δ*Cys* (○) gene. Gap1 activities were calculated in nanomoles per minute per milliliter to avoid a dilution effect due to NH_4^+-induced arrest of Gap1 synthesis. (B) Immunoblot of Gap1 from crude extracts prepared from the above cited strains before $(NH_4)_2SO_4$ addition (t_0) and at intervals thereafter. (C) Immunoblot of Gap1 from membrane-enriched extracts prepared from the above cited strains before $(NH_4)_2SO_4$ addition (t_0) and at intervals thereafter. The *npi1* alleles used to transforme the *npi1* mutant are indicated.

mutant, where Gap1 is resistant to NH_4^+-triggered down-regulation but still binds ubiquitin [3]. On the basis of such observations, it has been proposed that complete conversion of Gap1 to ubiquitinated forms occurs only if Gap1 is progressively removed from the plasma membrane by endocytosis [3].

In conclusion, we show that substitution of the invariant HECT-domain cysteine of the Npi1 ubiquitin

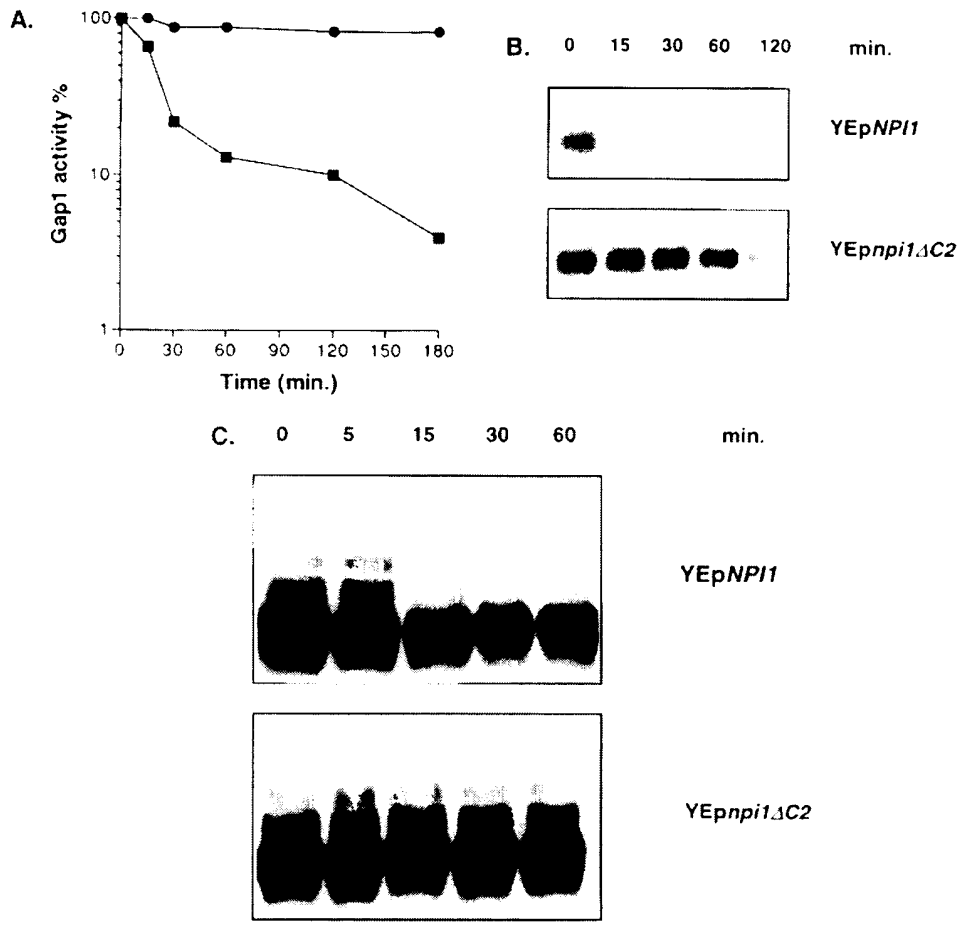

FIG. 3. Ubiquitination and down-regulation of Gap1 permease in cells overexpressing the wild-type *NPI1* or mutant *npi1ΔC2* gene. (A) The activity of Gap1 was measured as described in the legend of Fig. 2. Strains: 27038a (*npi1*) transformed with the high-copy-number plasmid pFL44S bearing the wild-type *NPI1* (■) or mutant *npi1ΔC2* (●) gene. (B) Immunoblot of Gap1 from crude extracts prepared from the above cited strains before $(NH_4)_2SO_4$ addition (t_0) and at intervals thereafter. (C) Immunoblot of Gap1 from membrane-enriched extracts prepared from the above cited strains before $(NH_4)_2SO_4$ addition (t_0) and at intervals thereafter. The *npi1* alleles used to transform the *npi1* mutant are indicated.

ligase, a residue involved in transient binding of ubiquitin before transfer to the target protein [12], impairs ubiquitination and NH_4^+-induced down-regulation of the Gap1 permease. We thus provide the first demonstration that this invariant cysteine is required for *in vivo* ubiquitination of a natural target protein. We also show that Npi1/Rsp5 ubiquitin ligase lacking its N-terminal C_2 domain is competent for Gap1 ubiquitination but not for Gap1 down-regulation. We cannot rule out a quantitative or qualitative defect in Gap1 ubiquitination in *npi1ΔC*$_2$ cells, but this seems unlikely, since the Gap1 ubiquitination profiles obtained in immunoblot experiments were similar for *NPI1*- and *npi1ΔC*$_2$-expressing cells. Furthermore, multiple copies of the *NPI1ΔC*$_2$ gene failed to restore sensitivity of Gap1 to NH_4^+ regulation. Our data argue, rather, for participation of Npi1/Rsp5 via its C_2 domain in endocytosis of ubiquitinated permease. Although a complete lack of Npi1/Rsp5 or replacement of its HECT-domain cysteine is incompatible with yeast cell viability, *npi1Δ* cells expressing Npi1ΔC_2 are viable. This is consistent with a specific role of the C_2 domain of Npi1/Rsp5 in endocytosis, an unessential function in yeast. Further experiments will be needed to determine the exact role of this domain in down-regulation of Gap1. The C_2 domain has been found in numerous proteins and shown in several of them to mediate interaction with membrane phospholipids, proteins, or inositol polyphosphates. Most C_2 domains also bind Ca^{++}, which either stimulates or modulates the specificity of binding to substrates [15]. For instance, an increase in cytosolic Ca^{++} in polarized MDCK epithelial cells causes the mouse Npi1/Rsp5 homologue Nedd4 to associate with apical and lateral membranes, this requiring the protein's N-terminal C_2 domain [21]. Our results showing that the Gap1 permease is still ubiquitinated in cells expressing Npi1ΔC_2 suggest correct targeting of the ubiquitin ligase. Perhaps the C_2

domain mediates targeting of Npi1 to regions of the plasma membrane that actively endocytose, or to some other specific membrane compartment. For instance, sorting of internalized Gap1 to the vacuole might require several Npi1-catalyzed ubiquitination steps in successive endosomal compartments, and a lack of Npi1/Rsp5 in one of these compartments could lead to Gap1 recycling to the plasma membrane. Ubiquitination of internalized protein for sorting to the lysosome rather than recycling to the plasma membrane has indeed recently been demonstrated in the case of a growth hormone receptor [22]. Alternatively, perhaps the C_2 domain of Npi1/Rsp5 mediates interaction with another protein, this interaction being essential to endocytosis. In keeping with this hypothesis, it is noteworthy that the two C_2 domains of synaptotagmin I interact respectively with clathrin AP-2 [23] and syntaxin [24]. Furthermore, recent evidence suggests that Npi1/Rsp5 is a component of the Pan1 complex proposed to coordinate several pathways (ubiquitin, actin, clathrin) implicated in endocytosis in yeast and mammalian cells [18]. Further experiments will be needed to determine the precise role(s) of Npi1/Rsp5 in the endocytotic system in yeast and mammalian cells.

ACKNOWLEDGMENTS

We are grateful to S. Kumar for providing mNedd4 antisera. This work was supported by the Fund for Medical Scientific Research (Belgium F.R.S.M. 3.4602.94) and the Communauté française de Belgique-Direction de la Recherche Scientifique–Action de Recherche Concertée. J.Y. S. was the recipient of a predoctoral fellowship from the Fond pour la formation à la Recherche dans l'Industrie et dans l'Agriculture (F.R.I.A.) and the Fondation Universitaire David et Alice Van buuren.

REFERENCES

1. Schandel, K. A., and Jenness, D. D.(1994) *Mol. Cell Biol.* **14,** 7245–55
2. Volland, C., Urban-Grimal, D., Géraud, G., and Haguenauer-Tsapis, R. (1994) *J. Biol. Chem.* **269,** 9833–9841.
3. Springael, J. Y. and Andre, B. (1998) *Mol. Biol. Cell* **9,** 1253–1263.
4. Riballo, E., Herweijer, M., Wolf, D. H., and Lagunas, R. (1995) *J. Bacteriol.* **177,** 5622–5627.
5. Horak, J., and Wolf, D. H. (1997) *J. Bacteriol.* **179,** 1541–1549.
6. Hochstrasser, M. (1996) *Annu. Rev. Genet.* **30,** 405–39, 405–439.
7. Terrell, J., Shih, S., Dunn, R., and Hicke, L. (1998) *Mol. Cell* **1,** 193–202.
8. Galan, J. and Haguenauer-Tsapis, R. (1997) *EMBO J.* **16,** 5847–5854
9. Galan, J.-M., Moreau, V., Andre, B., Volland, C., and Haguenauer-Tsapis, R. (1996) *J. Biol. Chem.* **271,** 10946–10952.
10. Medintz, I., Jiang, H., and Michels, C. A. (1998) *J. Biol. Chem.* **273,** 34454–34462.
11. Hein, C., Springael, J.-Y., Volland, C., Haguenauer-Tsapis, R., and Andre, B. (1995) *Mol. Microbiol.* **18,** 77–87.
12. Huibregtse, J. M., Scheffner, M., Beaudenon, S., and Howley, P. M. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 2563–2567.
13. Staub, O., Dho, S., Henry, P. C., Correa, J., Ishikawa, T., McGlade, J., and Rotin, D. (1996) *EMBO J.* **15,** 2371–2380.
14. Staub, O., Gautshi, I., Ishikawa, T., Breitschopf, K., Ciechanover, A., Schild, L., and Rotin, D. (1997) *EMBO J.* **16,** 6325–6336
15. Rizo, J., and Sudhof, T. C. (1998) *J. Biol. Chem.* **273,** 15879–15882.
16. Sudol, M. (1994) *Oncogene* **9,** 2145–2152.
17. Zolladek, T., Tobiasz, A., Vaduva, G., Boguta, M., Martin, N. C., and Hopper, A. K. (1997) *Genetics* **145,** 595–603.
18. Wendland, B., and Emr, S. D. (1998) *J. Cell Biol.* **141,** 71–84.
19. Benedetti, H., Raths, S., Crausaz, F., and Riezman, H. (1994) *Mol. Biol. Cell* **5,** 1023–1037.
20. Kumar, S., Harvey, K. F., Kinoshita, M., Copeland, N. G., Noda, M., and Jenkins, N. A. (1997) *Genomics* **40,** 435–443.
21. Plant, P. J., Yeger, H., Staub O., Howard P., and Rotin, D. (1997) *J. Biol. Chem.* **272,** 32329–32336.
22. Levkowitz, G., Waterman, H., Zamir, E., Kam, Z., Oved, S., Langdon, W. Y., Beguinot, L., Geiger, B., and Yarden, Y. (1998) *Genes Dev.* **12,** 3663–3674.
23. Zhang, J. Z., Davletlof, B. A., Südhof, T. C., and Anderson, R. G. W. (1994) *Cell* **78,** 751–760.
24. Li, C., Ullrich, B., Zhang, J. Z., Anderson, R. G., Brose, N., and Sudhof, T. C. (1995) *Nature* **375,** 594–599.
25. Béchet, J., Grenson, M., and Wiame, J.-M. (1970) *Eur. J. Biochem.* **12,** 31–39.
26. Springael, J. Y., Galan, J. M., Haguenauer-Tsapis, R., and André, B. (1999) *J. Cell Sci.* (in press).
27. Singer-Kruger B, Nemoto Y, Daniell L, Ferro Novick S, and De Camilli, P. (1998) *J. Cell Sci.* **111,** 3347–3356.
28. Wang, G., Yang, J., and Huibregtse, J. M. (1999) *Mol. Cell Biol.* **19,** 342–352.